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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/490,609	01/25/2000	Roderick T. Bunch	SO-3170	7385

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EXAMINER	
ZARA, JANE J	
ART UNIT	PAPER NUMBER
1635	

DATE MAILED: 08/10/2004

Please find below and/or attached an Office communication concerning this application or proceeding.

3/29

Office Action Summary	Application No.	Applicant(s)
	09/490,609	BUNCH ET AL.
	Examiner	Art Unit
	Jane Zara	1635

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *25 May 2004*.

2a)☒ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *25-28 and 31-33* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☒ Claim(s) *31-33* is/are allowed.

6)☒ Claim(s) *25-28* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☐ Other: _____.

DETAILED ACTION

This Office action is in response to the communication filed 5-25-04.

Claims 25-28 and 31-33 are pending in the instant application.

Response to Arguments and Amendments

Withdrawn Rejections

Any rejections not repeated in this Office action are hereby withdrawn.

Maintained Rejections

Claims 25-28 are rejected under 35 U.S.C. 102(e) as being anticipated by Ecker for the reasons of record set forth in the Office action mailed 2-25-04.

Applicants' arguments filed 5-25-04 have been fully considered but they are not persuasive.

Applicants argue that Ecker does not anticipate the claimed invention because nothing in the reference teaches specific nucleic acid hybridization and use of the 280 and 488 nucleic acid sequences as biomarkers. The claimed invention is drawn to methods for determining a level or pattern of carcinogenesis biomarker in a cell in vitro comprising detection of hybridization to SEQ ID NO: 280 or 488. Contrary to Applicants' assertions, Ecker teaches the ability to detect SEQ ID NO: 280 (a.k.a. regucalcin or senescence marker protein 30, see Table 1, col. 9-10 of Ecker) mRNA transcripts by hybridization (see col. 1, lines 24-36), whose increased expression is used as an indicator of a cancer phenotype (see col. 1-2 of Ecker). The title of Ecker's patent is the *Identification*

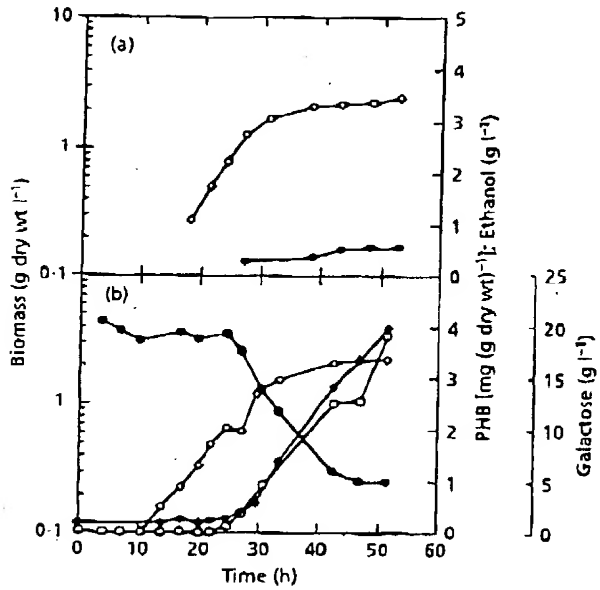

Fig. 2. PHB synthesis in (a) plasmid-free host strain D603 and (b) recombinant D603(pTL85) cultivated in bioreactors with galactose as sole carbon source. Biomass concentration (○), specific PHB content (●), galactose concentration (●) and ethanol concentration (○) are shown.

is truncated. The PHB synthase gene is known to be insensitive to moderate truncation at its N-terminus. Schubert *et al.* (1991) reported that a truncated gene lacking the first 107 nucleotides produces fully active synthase when expressed in *E. coli*, and that a gene lacking the first 299 nucleotides produced enzyme having 7% of the full protein activity. In addition, PHB accumulation was induced in *E. coli* in our lab by a plasmid carrying a PHB operon with a glutathione *S*-transferase/synthase fusion gene lacking the first 210 nucleotides of the synthase. It was therefore expected that the truncated synthase gene in pTL85, lacking the first 140 nucleotides, would give rise to functional enzyme.

Yeast strains D603 and YPH500 were transformed with the PHB synthase plasmid pTL85 and tested for their ability to synthesize PHB. As an initial screening, cells were grown in shake flasks on SD medium and then assayed for PHB. In all media the initial sugar concentration was 20 g l^{-1}, so that sugar was not the limiting nutrient (DaSilva, 1988; Fig. 2). ODs of the cultures were monitored and cells were harvested for analysis after 15–20 h into stationary phase. This corresponds to a culture age of 47 h with glucose as carbon source, 64 h with galactose as carbon source and 93 h with galactose medium containing compactin. All flasks were grown in the same incubator-shaker, and in each case three separate flasks were inoculated from the same seed culture and PHB content determined separately for each flask. Data from shake flasks indicated that plasmid-free cells did not contain measurable PHB, nor did plasmid-containing cells grown under non-inducing conditions (glucose as sole carbon source). In contrast, plasmid-containing cells grown under inducing conditions contained PHB in the order of 1 mg PHB (g dry wt)$^{-1}$. The degree to which PHB accumulated in recombinant cells seemed to have some dependence on host strain, as evidenced by the difference between PHB levels produced in strains D603(pTL85) [1·4 mg PHB (g dry wt)$^{-1}$] and YPH500/pTL85 [0·31 mg PHB (g dry wt)$^{-1}$]. The presence of 28 mg compactin l^{-1}, an enzyme inhibitor discussed further below, seemed to cause a threefold increase in the PHB level attained by D603(pTL85) in stationary phase [3·9 mg PHB (g dry wt)$^{-1}$]. Coefficients of variation for these measurements were typically 20%.

The results verify the functionality of the PHB synthase protein expressed from the plasmid. Evidence that PHB synthase requires a phosphopantetheine prosthetic group for catalytic activity (Gerngross *et al.*, 1994) suggests that the yeast cell is able to carry out this post-translational modification on this heterologous protein. PHB synthesis also implies that D-3HB-CoA, the substrate for PHB synthase, is somehow formed in the cells without the presence of the other PHB synthetic enzymes of *A. eutrophus*, a process that does not seem to occur in other cell types. Expression of the synthase gene alone in *E. coli* produces no PHB (Peoples & Sinskey, 1989). Transgenic *Arabidopsis thaliana* plants expressing only a synthase gene similarly do not produce PHB (Poirier *et al.*, 1992). These results do not rule out the possibility that the necessary auxiliary enzymes exist in these host cells but were not expressed under the conditions used for study.

The shake flask results must be interpreted with caution, since they do not reveal whether PHB synthesis occurred during growth or, as is observed for *A. eutrophus*, during non-growth conditions. These experiments were followed with batch culture in bioreactors, focussing on strain D603(pTL85) as it appeared to accumulate the greatest amounts of PHB.

Kinetics of PHB formation in yeast

The pattern of PHB accumulation in yeast over the course of batch growth was followed during controlled culture in a bioreactor. The recombinant as well as the plasmid-free host strain were grown aerobically at 30 °C and pH 4·5 in bioreactors on enriched SD medium containing initially 20 g galactose l^{-1}, lacking uracil where appropriate. This medium was nitrogen-limited, with galactose levels near 5 g l^{-1} when the cells reached stationary phase. The cultures were sampled over the course of batch growth for PHB and protein analysis.

Representative data from bioreactor runs are shown in Fig. 2. The untransformed host strain generated a GC signal corresponding to a PHB content of 0·2–0·5 mg PHB (g dry wt)$^{-1}$. This level is about two orders of magnitude higher than that reported by Reusch (1989) for wild-type *S. cerevisiae* cells, and about one order of magnitude lower than that reported by Nuti & Lepidi

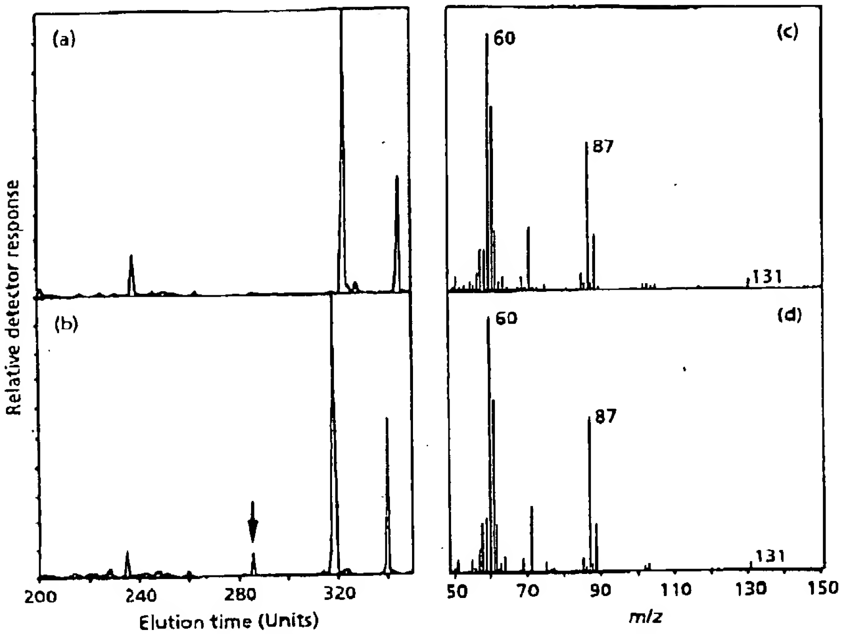

Fig. 3. GC/MS analysis of cell extracts, indicating the presence of PHB in recombinant yeast. Dried cell material was subjected to propanolysis and solvent extraction. Comparison of GC traces of (a) host strain D603 and (b) recombinant D603(pTL85) shows a peak (arrow) corresponding to the same elution time as the propyl 3HB standard (not shown). MS analysis of (c) a propyl 3HB reference sample and (d) the material suspected to be propyl 3HB in the recombinant cell extract confirms the identity of the GC peak in (b).

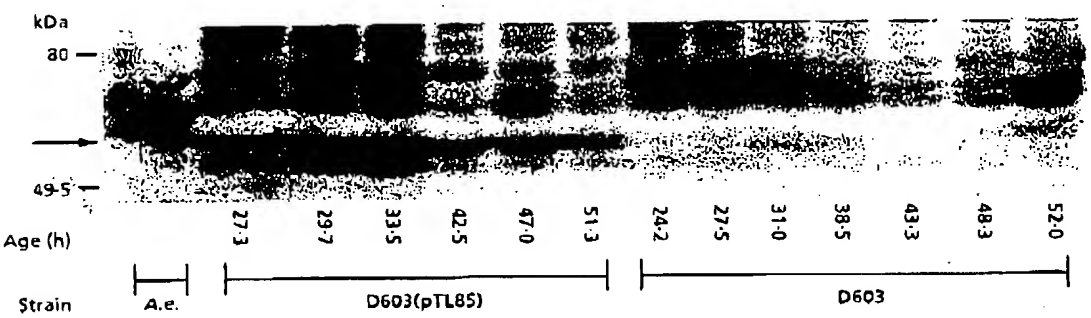

Fig. 4. Scanned image of a Western blot of proteins from yeast strains D603 and D603(pTL85). The *A. eutrophus (A.e.)* protein is included as a reference. The position of the putative truncated synthase produced by the recombinant is indicated by the arrow. All yeast proteins were loaded onto the gel at 100 μg per lane, *A. eutrophus* protein at 50 μg.

(1974). Identification of GC peaks as propyl 3HB was made using GC/MS (Fig. 3).

In recombinant D603(pTL85), PHB content was essentially equal to wild-type levels during exponential growth but a definite accumulation of PHB occurred in the stationary phase. The final PHB level in recombinant cells represents a 10-fold increase as compared to plasmid-free cells when grown on galactose as the sole carbon source. Levels of PHB at an extended cultivation time of 120 h approached 6 mg PHB (g dry wt)$^{-1}$ for the recombinant (data not shown). During the accumulation phase, the maximum rate of PHB synthesis was about 2·5 μg PHB (g dry wt)$^{-1}$ h^{-1} or 0·03 μmol PHB monomers (g dry wt)$^{-1}$ h^{-1}. This rate of PHB synthesis, on a per unit non-PHB biomass basis, is about 10^5 times less than that observed during PHB accumulation in *A. eutrophus* (Pedrós-Alió *et al.*, 1985; Heinzle & Lafferty, 1980). Interestingly, PHB accumulation coincided with ethanol production, suggesting that the same metabolic conditions (e.g., availability of carbon and an excess of reducing equivalents) favour both processes.

Western blot identifies truncated PHB synthase

Western blots were performed to qualitatively evaluate levels of truncated synthase protein. Cell samples for protein analysis were collected from the reactor runs depicted in Fig. 2. An image of a Western blot prepared from crude cell-free extracts is shown in Fig. 4. Extracts from D603(pTL85) reveal a band near 60 kDa, the size of the putative truncated protein, that does not appear in extracts from the plasmid-free host strain. This band

appears to weaken as cells progress through the PHB accumulation phase. A possible explanation for this is that the synthase becomes associated with PHB granules and is removed from the soluble fraction during preparation of protein extracts (Nawrath *et al.*, 1994).

Since antibodies were raised against a truncated synthase, the intensity of Western blots should reflect molar protein concentrations for both full-length and truncated synthases. Densitometry on the Western blot suggests that the recombinant yeast cells contain up to 30%, on a per unit protein basis, of the molar concentration present in *A. eutrophus* cells. This seems high in light of the very low rate of PHB synthesis observed in recombinant yeast. Although a number of factors may affect the densitometry quantification, the results suggest that enzyme activities other than the synthase are limiting the rate of synthesis of PHB. Compartmentation of pathway enzymes or precursors could also affect rates of PHB synthesis.

^{1}H-NMR identifies PHB

Solvent extracts of yeast cells prepared as described were subjected to ^{1}H-NMR (Fig. 5). For strain D603, 24·7 g dry cell material containing 0·19 mg PHB (g dry wt)$^{-1}$ (by GC analysis) yielded 29 mg methanol-precipitated material for ^{1}H-NMR. The ^{1}H-NMR spectrum obtained from this extract (Fig. 5c) failed to show either PHB or 3HB. For the recombinant strain D603(pTL85), 14·7 g dry cell material containing 4·0 mg PHB (g dry wt)$^{-1}$ (by GC analysis) yielded 10 mg methanol-precipitated material. ^{1}H-NMR of this material (Fig. 5d) showed the characteristic methylene and methine proton resonances found in PHB (Fig. 5a). The monomer 3HB (free acid) displays a methine proton resonance with a significantly lower chemical shift (Fig. 5b), and is not apparent in extracts from either wild-type or recombinant cells. Our conclusions from ^{1}H-NMR data are that the recombinant yeast cells do in fact synthesize polymer, while the wild-type cells appear to contain no polymer.

The methodology used for PHB detection deserves some reflection. Most researchers have analysed PHB using techniques in which it is not the polymer but a derivatized product that is detected. Examples of this are the method of Law & Slepecky (1961) where concentrated sulfuric acid induces conversion of the polymer to crotonic acid, and the method of Riis & Mai (1988), used in this study, in which reaction with acidified propanol forms propyl 3HB. The drawback to these chemical methods is the likelihood that non-polymeric moieties such as 3HB or its CoA thioester will derivatize along with the polymer and distort the measurement.

Fortunately, it is possible to unambiguously distinguish between polymer and monomeric species using ^{1}H-NMR. Problems with this technique are the relatively large amount of material required as compared to chemical methods, and interference by contaminants in cell extracts. A survey of some cell types not known to accumulate PHB (Seebach *et al.*, 1994), yeast not included, demonstrated by ^{1}H-NMR that the polymer was in fact present.

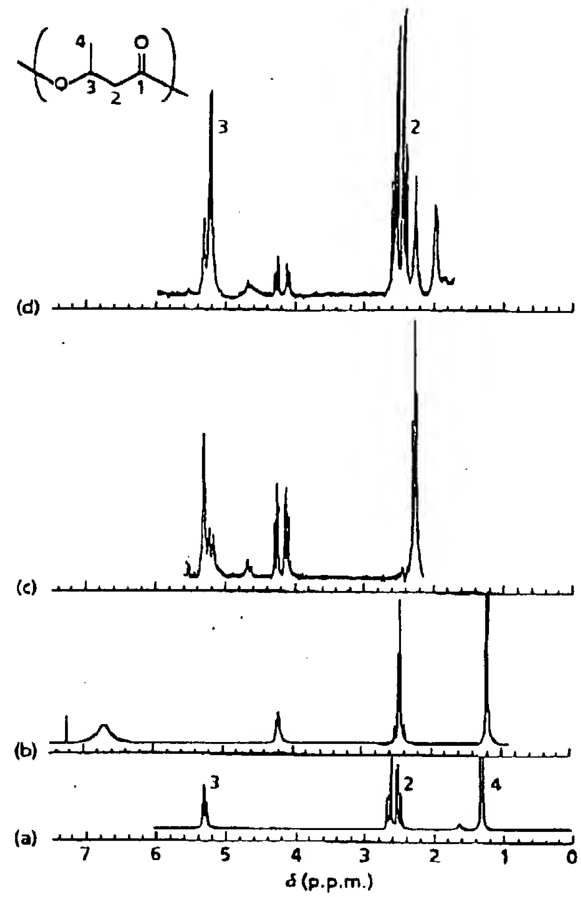

Fig. 5. **^{1}H-NMR spectra for (a) PHB, (b) 3-hydroxybutyric acid, (c) chloroform-extracted, methanol-precipitated material from wild-type yeast and (d) recombinant D603(pTL85). PHB proton resonances are clearly visible in the recombinant (d). All samples were dissolved in d-chloroform. The PHB structure with numbered carbon atoms is represented at the upper left.**

These authors also discussed difficulties in detecting the small amounts of PHB, with the requirement for gel chromatographic purification to obtain satisfactory ^{1}H-NMR spectra from some samples.

PHB in wild-type yeast was originally reported by Reusch (1989) and to our knowledge has never been confirmed by ^{1}H-NMR (Reusch, 1992; Seebach *et al.*, 1994). These authors have not attempted monomer detection by ^{1}H-NMR, though such species may be relatively insoluble in chloroform. Although we could not detect polymer in our wild-type strains, questions remain about ^{1}H-NMR detection limits. The wild-type ^{1}H-NMR sample, from which no PHB signal was detected, contained only 45 μg PHB as indicated by the GC assay. Questions also remain about the extraction method employed. Reusch (1992) suggests that native low molecular mass PHB may be complexed with various lipids or proteins, making its